Retinal Neovascularization Is Suppressed With a Matrix Metalloproteinase Inhibitor

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Objectives: To determine the role of extracellular proteinases in ischemia-induced retinal neovascularization in an animal model and to examine the effect of proteinase inhibitors on retinal neovascularization.

Methods: Retinal neovascularization was induced in newborn mice exposed to 75% oxygen for 5 days, followed by room air. Retinal extracts underwent zymographic analysis to measure the activity of urokinase and matrix metalloproteinases (MMPs). Some animals under the same conditions also received intraperitoneal injections of an MMP inhibitor. Histological analysis was done to quantitate the neovascular response in these animals.

Results: Levels of urokinase and MMPs (MMP-2 and MMP-9) in retinas were significantly increased in animals with induced retinal neovascularization. Neovascularization was significantly inhibited with intraperitoneal administration of an MMP inhibitor.

Conclusion: Systemic inhibition of MMPs may have therapeutic potential in preventing retinopathy associated with retinal neovascularization.

Clinical Relevance: Because up-regulation and activation of proteinases represents a final common pathway in the process of retinal neovascularization, pharmacological intervention of this pathway may be an alternative therapeutic approach to proliferative retinopathy.


Retinal neovascularization is a leading cause of blindness in a variety of clinical conditions, including diabetic retinopathy, retinopathy of prematurity, and retinal vein occlusion. Left untreated, these conditions can result in intraocular hemorrhage and retinal detachment leading to severe visual loss. Current laser treatment for these diseases, although successful in slowing the growth of new vessels, is not optimal. This treatment may result in the loss of peripheral and night vision, and the disease may progress despite treatment. It is well accepted that hypoxia occurs in these clinical conditions and leads to an initiation of the angiogenic process in the retina. A balanced interplay of proteinases and proteinase inhibitors has been implicated in the process of angiogenesis and has been extensively studied during the development of tumor angiogenesis.

The objective of this study was to determine the role of proteinases in ischemia-induced retinal neovascularization in an animal model, namely, newborn mice exposed to the variable oxygen cycle. This model system closely resembles retinopathy of prematurity and some of the characteristics seen in proliferative diabetic retinopathy, such as capillary dropout and neovascularization of the optic disc. We also investigated the effect of a proteinase inhibitor on retinal neovascularization in this model.

RESULTS

Hyperoxia (75% oxygen) followed by room air treatment led to a quantifiable neovascular response in 100% of the exposed animals. Among which vascular endothelial growth factor (VEGF) is currently thought to be the major mediator of neovascularization.

One phase of the angiogenic process is the invasion and migration of microvascular endothelial cells through the capillary basement membrane and into the adjacent extracellular matrix. This invasive process is tightly coupled to the production and activity of specific extracellular proteinases, including the serine proteinase urokinase and specific members of the matrix metalloproteinase (MMP) family. A balanced interplay of proteinases and proteinase inhibitors has been implicated in the process of angiogenesis and has been extensively studied during the development of tumor angiogenesis.

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in room air for 17 days served as controls and showed no evidence of neovascularization extending into the vitreous beyond the inner limiting membrane. Quantification revealed a significant increase in retinal neovascularization in animals treated with 75% oxygen and verified the previously established model of experimentally induced retinal neovascularization (Table 1).12,13

Results of zymographic analyses of retinal extracts from animals on day 17 (the active angiogenic phase) revealed significant increases in the high (54-kd) and low (32-kd) molecular weight forms of urokinase compared with controls (Table 2). Significant increases were also found in the levels of proenzyme (72-kd) and active (62-kd) forms of MMP-2. Analysis of messenger RNA did not detect expression of MMP-3 and MMP-7 in either control or experimental animals, confirming the results of zymographic analysis (Figure 2, A).

We next investigated the effect of a synthetic MMP inhibitor on the development of retinal neovascularization in this animal model. Results of analysis of neovascular nuclei count on histological examination revealed a 72% reduction in retinal neovascularization with the intraperitoneal administration of BB-94, 1 mg/kg, compared with animals receiving intraperitoneal injection of saline solution as a placebo (Figure 1, C and D, and Table 1). No obvious toxic effects, inflammation, or abnormal retinal neuronal or vascular development was detected in animals receiving BB-94, 1 mg/kg, based on the light microscopic histological appearance of the tissue. In addition, analysis of retinal sections stained with an endothelial cell–specific antibody (anti-CD 31) demonstrated that the number and distribution of capillar-
ies within the retina in the drug-treated group was similar to that of control animals. The number of capillaries per section was 8.0 ± 2.3 in the drug-treated group vs 6.6 ± 2.8 in the control group (P = .05). However, some animals died after receiving a single injection of the high dose (15 mg/kg) of BB-94. These effects may have been caused by an inhibition of MMP that potentially play an important role in neuronal maturation occurring in these animals during this time.

Oxygen-exposed animals without any treatment gained an average of 1.5 g in weight (27.3%) from day 12 to day 17, whereas those treated with BB-94 gained an average of 1.3 g in weight (21.3%) during the same time. Oxygen-exposed animals injected with isotonic sodium chloride had an average weight gain of 1.2 g (22.5%), which was not significantly different from the latter groups. Thus, the possibility of any nonspecific effect of the drug on retinal neovascularization, secondary to weight loss or slower rate of weight gain from toxic effects of using the drug, was ruled out in this experiment.

Significant decreases in the level of active species of MMP-2 and MMP-9 were seen in response to BB-94 treatment when retinal extracts from drug-treated animals and controls were compared (Table 2). In addition to its ability to inhibit the function of the active forms of MMP-2 and MMP-9, BB-94 may also prevent the activation of their proenzyme forms. For MMP-2 and MMP-9, conversion of the proenzyme form of the protein has been shown to be partially dependent on the activity of MMPs.

**COMMENT**

Our results show that the expression of proteinases is increased during the retinal neovascularization process. The production of urokinase and specific members of the MMP family are significantly elevated in retinal extracts from mice with active neovascularization. These results correlate well with data obtained from examining proteinases in epiretinal neovascular membranes that were surgically removed from humans with proliferative diabetic retinopathy.

A common mechanism in mouse retinal neovascularization and human proliferative diabetic retinopathy may be an initiating hypoxic event followed by an increased expression of angiogenic proteins, including VEGF. Either or both of these mechanisms (hypoxia and VEGF) may affect the subsequent expression of proteinases by microvascular cells. Results of a previous study demonstrate that isolated retinal capillary endothelial cells selectively up-regulate and activate the MMP-2 enzyme under hypoxic conditions and in response to VEGF stimulation. This is interesting in light of the present findings and suggests that interactions occur in intact retina that...
result in protease expression, other than MMP-2, by vascular cells. Alternatively, retinal capillaries may not be the sole source of these enzymes in the retina. In addition, it is not known whether protease expression by microvascular endothelial cells occurs as a direct response to hypoxic conditions or whether it is mediated through the production of VEGF or other factors.

Extracellular proteases are essential during the invasive stage of the angiogenic process in facilitating the degradation of the capillary basement membrane and the subsequent invasion of activated endothelial cells into surrounding tissues. In addition to its role in matrix degradation through the production of plasmin, urokinase has been shown to affect the motile behavior of cells by regulating cell-matrix interactions. Of significance in this study was the finding of an increase in the high and low molecular weight forms of urokinase in retinas exhibiting neovascularization. The increased low molecular weight form suggests the presence of an amino terminal fragment of urokinase in this tissue. This protein fragment contains a growth factor–like domain and has been suggested to play a role in the stimulation of cell proliferation, another important event in the angiogenic process.

MMP-2 has also been shown to play an important role in the interactions, which take place between a motile cell and its substratum. In angiogenic blood vessels in particular, MMP-2 has been shown to interact with the αvβ3 integrin on the endothelial cell surface to create localized areas of high proteolytic activity. This interaction has been postulated to facilitate the generation of extracellular matrix protein fragments that serve as substrates for the αvβ3 integrin and lead to increased cell survival and matrix invasion. Results of these studies and our own lead us to speculate that the decrease in angiogenic response seen in the retina after administr-
Figure 2. Reverse transcription polymerase chain reaction analysis of matrix metalloproteinases and urokinase. A, First-strand complementary DNA was synthesized from total RNA extracted from the retinas of control (lanes 1-6) and experimental (lanes 7-12) mice (retinas pooled from 5 animals on postnatal day 17). Complementary DNA was used in standard polymerase chain reactions with primers specific for MMP-2 (lanes 1 and 7), MMP-3 (lanes 2 and 8), MMP-7 (lanes 3 and 9), MMP-9 (lanes 4 and 10), MT-MMP (lanes 5 and 11), and the 18s ribosomal RNA as an internal control (lanes 6 and 12). The identity of the polymerase chain reaction products was confirmed by cloning into the pCR2.1 vector followed by sequencing. B, Relative reverse transcription polymerase chain reaction analysis of MMP-2, MMP-9, and MT-MMP in retinas from control and experimental mice (n = 3). Significant increases were seen in the relative amount of messenger RNA for each of these proteinases in animals with retinal neovascularization compared with control animals. As seen in (A), neither control nor experimental animals showed expression of MMP-3 or MMP-7 in the retina. Asterisk indicates significantly different at P<.01.

Table 2. Zymographic Analysis of Proteinases Present in the Retina of Mice Exhibiting Ischemia-Induced Neovascularization∗

<table>
<thead>
<tr>
<th>Group</th>
<th>Drug Treatment</th>
<th>Eyes, No.</th>
<th>MMP-2 72 kd</th>
<th>MMP-2 62 kd</th>
<th>MMP-2 92 kd</th>
<th>MMP-2 84 kd</th>
<th>MMP-2 54 kd</th>
<th>MMP-2 32 kd</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>None</td>
<td>12</td>
<td>0.25 ± 0.04</td>
<td>0</td>
<td>1.2 ± 0.34</td>
<td>0</td>
<td>10.4 ± 1.1</td>
<td>3.9 ± 0.55</td>
</tr>
<tr>
<td>Experimental</td>
<td>None</td>
<td>12</td>
<td>0.94 ± 0.19†</td>
<td>0.53 ± 0.11†</td>
<td>5.3 ± 0.89†</td>
<td>1.6 ± 0.33†</td>
<td>29.8 ± 2.5†</td>
<td>17.2 ± 1.6†</td>
</tr>
<tr>
<td>Experimental</td>
<td>BB-94, 1 mg/kg</td>
<td>12</td>
<td>0.73 ± 0.09</td>
<td>0.24 ± 0.06‡</td>
<td>1.3 ± 0.19</td>
<td>0‡</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Experimental</td>
<td>BB-94, 15 mg/kg</td>
<td>4</td>
<td>0.28 ± 0.02‡</td>
<td>0.13 ± 0.02‡</td>
<td>1.2 ± 0.54</td>
<td>0‡</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

*Values are given as pixels per microgram of DNA, mean ± SEM. Retinal neovascularization was induced in C57Bl/6J mice as described, and the retinas were collected and analyzed for the presence of matrix metalloproteinases and urokinase. Some mice were injected with either 1 or 15 mg/kg of the matrix metalloproteinase inhibitor BB-94 before or during the angiogenic period. ND indicates not done.
†Significantly different from controls at the P<.001 level.
‡Significantly different from experimental (non–drug-treated) mice at the P<.001 level.

The current clinical treatment for proliferative retinopathy is laser photocoagulation. Despite the effectiveness of this therapy, the disease process may progress or recur and often results in adverse effects, including the loss of peripheral and night vision. Treatment options for retinopathy would therefore benefit from an investigation into the use of alternative therapies. Initial attempts to develop a suitable therapy have targeted primarily the VEGF system through the use of neutralizing antibodies, chimeric proteins, or antisense molecules. Other studies have used inhibitors of protein kinase Cβ and growth hormones. The present study used an MMP inhibitor at a dose of 1 mg/kg to reduce retinal neovascularization by 72%. Further suppression using higher doses was achieved but with toxic effects to some animals. Future studies will use a nonsystemic, topical application of higher doses of BB-94 to inhibit the development of new vessels and to avoid toxic effects of the drug. Because the activity of urokinase was also increased in retinas in this animal model, studies using urokinase inhibitors to suppress retinal neovascularization are currently in progress. Because up-regulation and activation of MMPs represent a final common pathway in the process of neovascularization, use of an inhibitor of these enzymes may have potential therapeutic benefit in the treatment of many proliferative retinopathy conditions. In addition, this type of pharmacological approach would be expected to alleviate the destructive adverse effects of the laser treatments presently in use.

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REFERENCES
A look at the past...

NGELUCCI examined bacteriologically twelve eyes enucleated after having caused sympathethic ophthalmia, and also portions of the iris removed by iridectomy from the eyes sympathetically inflamed. From the detailed reported results of his inoculations, it is particularly worthy of note that the cocci and diplococci from the iris of the two eyes sympathetically inflamed caused similar inflammatory changes when inoculated into the eyes of rabbits.